Culture of very young *Phaseolus vulgaris* L. pods and plantlet regeneration

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Introduction

First results on the development of an *in vitro* pod culture technique for young pods of *P. vulgaris* (PV) are reported by Geerts *et al.* (2000). Different culture techniques for 2-day old *Phaseolus* pods were described using a modified Phillips *et al.* (1982) medium for maturation. The authors showed that the application of high and variable osmolality conditions, similar to those observed *in vivo*, during pod culture and before extracting the embryos, gave the best results in terms of ovule and embryo development (Geerts *et al.*1999). However, the plantlet survival remained very low and no regeneration was described. In this paper, we report the method used to obtain the first regeneration of plantlets from 2 days-old *Phaseolus* embryos using an *in vitro* pod culture technique.

Material and methods

A *P. vulgaris* genotype (NI 637) was grown in a growth chamber under the following controlled conditions: day/night temperature of 24/20°C, light intensity of 580 $\mu mol.m^{-2}s^{-1}$ and a day length of 11 h 30 min. Pod culture technique was adapted from Geerts *et al.* (2000). Basal medium contains Phillips *et al.* (1982) mineral salts, 80 $g.l^{-1}$ sucrose, 1 $mg.l^{-1}$ thiamin HCl, 5 $mg.l^{-1}$ nicotinic acid, 0.5 $mg.l^{-1}$ pyridoxine, 100 $mg.l^{-1}$ myoinositol, 1,000 $mg.l^{-1}$ L-glutamine, 1,000 $mg.l^{-1}$ casein hydrolysate, 0.1 µM NAA, 10 µM adenine and 1.0 µM Benzylaminopurine. Seven pod culture methods were compared using three different osmoticum agents (Table I): four solid media (S1, S2, S3 and S4) and three liquid media (L1, L2 and L3). For each of the solid cultures, a sequence of three media with a decreasing osmotic pressure were used. Pods were cultured on 580 mosm of osmolality during 1 day, transferred to 450 mosm for 2 days more, and lastly transferred for 4 days more to basal medium (350 mosm). For S4 technique, pods were directly transferred to basal medium for 1 week.

Table I. Type and quantity of osmoticum added to the basal medium.

Techniques	***Osmoticum added***	***Osmolalities of media***		
		580 mosm (*)	***450 mosm***	***350 mosm***
S1(**) - L1	Sucrose	63 $g.l^{-1}$	22 $g.l^{-1}$	-
S2 - L2	Mannitol	36 $g.l^{-1}$	16 $g.l^{-1}$	-
S3 - L3	Polyethylene glycol 8000	128 $g.l^{-1}$	55 $g.l^{-1}$	-
S4	-	-	-	-

(*) at this osmolality, vitamins, amino acids and growth regulators of basal medium are modified : 0.25 $mg.l^{-1}$ thiamin HCl, 1.25 $mg.l^{-1}$ nicotinic acid, 0.125 $mg.l^{-1}$ pyridoxine, 25 $mg.l^{-1}$ myoinositol, 250 $mg.l^{-1}$ L-glutamine, 250 $mg.l^{-1}$ casein hydrolysate, 0.095 µM abscisic acid, 5.5 µM Tryptophan and 0.1 µM NAA.
(**) 7 $g.l^{-1}$ Select Agar-Gibco BRL.

For the culture on liquid media, pods were supported on sterilized glass beads in a Petri dish containing 100 ml of liquid Medium I. Petri dish was connected on the top-center by a peristaltic pump to a 1 liter bottle containing the basal liquid culture medium without complementary addition of osmoticum. During the first five days of culture, 100 ml per day were dripped from the 1 L bottle in the Petri dish, permitting a constant evolution of the culture medium osmolality. After 5 days the osmolality of the liquid medium reached 350 mosm.

After 1 wk of pod culture, embryos were extracted from ovules reaching at least 2 mm and transferred to basal solid medium for 2 wk in order to continue the maturation process. Mature embryos were transferred to a dehydration medium for 2 wk (Hu and Zanettini, 1995) before culturing them on a pre-germination medium containing the salts of Gamborg *et al.* (1968) and 1 µM Acid Indol Acetic (AIA) for 1 wk. Eventually, embryos were transferred to a germination medium containing the salts of Gamborg *et al.* (1968) but without AIA.

3. Results and discussion

Results are given in Table II. Significant differences between media were observed, mainly for pod growth (P = 0.019), number of ovules (P = 0.040) and number of extracted embryos (P = 0.013) per pod. In general, pod growth was higher on liquid media compared to solid media, reaching 90.1 % on L1 media. However, pod growth was not correlated with the development of ovules and embryos. The number of pods containing at least one developed ovule was not significantly different in liquid and solid media (55 % of the pods on L1 compared to 48 % on S1). Moreover, the number of ovules longer than 2 mm per pod was the highest on S1 medium with a mean of 3.7 developed ovules per pod compared to a mean of 2 developed ovules on L1 medium. Finally, the number of extracted embryos was also lower on L1 medium with only 1.9 extracted embryos per pod compared to 3.8 embryos on S1 medium.

While the development stage of extracted embryo was quite variable ranging from globular to cotyledonar, the mean embryo length was however higher when extracted from pods cultured on liquid media.

Finally, modifications of regeneration protocol described by Geerts *et al.* (2000), i.e. transfer of the embryos on the dehydration medium as proposed by Hu and Zanettini (1995) during two wk instead of one, and the further transfer during one week to a medium containing AIA, have increased significantly the acclimatization rate from 3 % in our previous results (Geerts *et al.*, 2000) to 90 % and permitted the regeneration of a few plantlets.

For the first time, adult plants of *Phaseolus* were regenerated from 2 days-old globular embryos thanks to the use of a device modifying the osmolality in the pod culture medium. This technique could provide an alternative solution to develop interspecific hybrids when embryos abort at very early stages.

Table II. Evaluation of the influence of seven culture conditions on *Phaseolus* pod, ovule and embryo growth and development.

Parameters	***Pod* in vitro *culture methods used***						
	S1	***S2***	***S3***	***S4***	***L1***	***L2***	***L3***
Total number of pods	181	24	16	67	87	46	28
Pod growth (in %)	43.9 ± 3.3	63.6 ± 4.8	28.1 ± 6.8	37.1 ± 5.6	90.1 ± 4.3	67.6 ± 6.2	73.8 ± 7.9
Nb of pods containing at least one developed ovule	87 (48 %)	15 (63 %)	2 (13 %)	29 (43 %)	48 (55 %)	20 (44 %)	12 (43 %)
Mean nb of developed ovules per pod	3.7 ± 0.2	2.1 ± 0.3	1.0 ± 0.0	2.6 ± 0.2	2.0 ± 0.2	3.5 ± 0.4	1.8 ± 0.5
Mean ovule length after 7 days pod culture (in mm)	1.6 ± 0.0	1.5 ± 0.1	1.7 ± 0.2	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	2.1 ± 0.1
Mean nb of extracted embryos per pod	3.8 ± 0.2	2.2 ± 0.3	1.0 ± 0.0	2.7 ± 0.2	1.9 ± 0.2	3.0 ± 0.3	1.7 ± 0.3
Mean embryo length at extraction (in µm) and corresponding stages(*)	16.9 ± 2.3 G to C	14.3 ± 3.8 G to HS	13.8 ± 12.5 G to EHS	16.5 ± 4.1 G to C	17.4 ± 2.7 G to C	17.5 ± 3.7 LG to C	18.8 ± 5.8 EHS to C
Embryo growth (in %)	94.2 ± 1.1	87.4 ± 3.8	99.0 ± 1.6	99.1 ± 1.2	98.8 ± 0.3	96.4 ± 0.2	-
Total nb of cultured embryos	82	12	2	24	35	12	-
Germination rate (in %)	60.7 ± 4.0	38.2 ± 12.1	50.0 ± 50.0	50.1 ± 8.5	30.8 ± 7.5	7.5 ± 5.1	-
Acclimatization rate (in %)	77.6 ± 4.3	100.0 ± 0.0	100.0 ± 0.0	76.0 ± 9.0	91.7 ± 6.0	100.0 ± 0.0	-

S1, S2, S3, S4 : solid media; L1, L2, L3 : liquid media; values are given with their standard error (± SE);
(*) G = globular, LG = late globular, EHS = early heart-shaped, HS = heart-shaped, C = cotyledonar.

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